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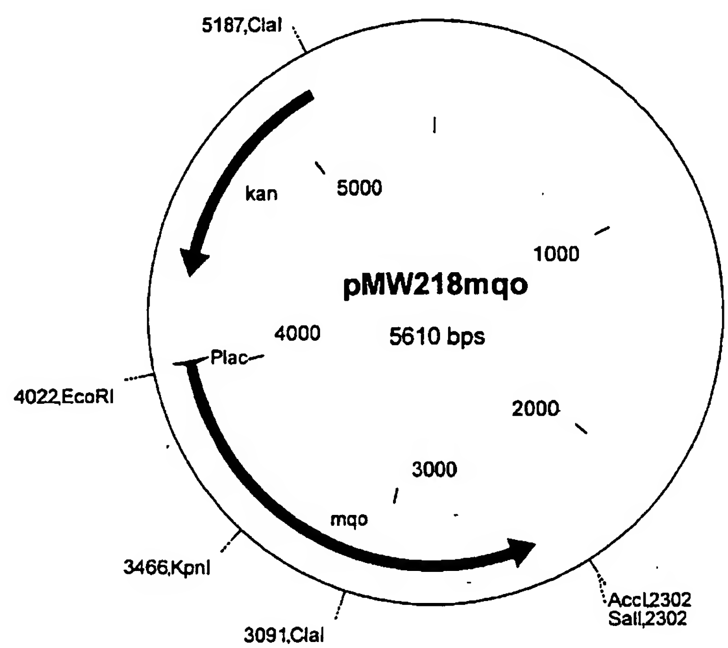
(54) **Title:** PROCESS FOR THE FERMENTATIVE PREPARATION OF L-THREONINE



(57) **Abstract:** The invention provides a process for the fermentative preparation of L-threonine using Enterobacteriaceae which in particular already produce L-threonine and in which the nucleotide sequence(s) which code(s) for the mqo gene are enhanced, in particular over-expressed.

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Process for the fermentative preparation of L-threonine

This invention relates to the new amino acid sequence of the malate:quinone oxidoreductase enzyme protein (Mqo) of Enterobacteriaceae and to a process for the fermentative preparation of L-threonine using Enterobacteriaceae in which the mqo gene is enhanced.

Prior Art

L-Threonine is used in animal nutrition, in human medicine and in the pharmaceuticals industry. It is known that L-threonine can be prepared by fermentation of strains of Enterobacteriaceae, in particular Escherichia coli and Serratia marcescens. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as e.g. stirring and supply of oxygen, or the composition of the nutrient media, such as e.g. the sugar concentration during the fermentation, or the working up to the product form by e.g. ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the threonine analogue α-amino-β-hydroxyvaleric acid (AHV), or are auxotrophic for metabolites of regulatory importance and produce L-threonine are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of Enterobacteriaceae strains which produce L-threonine, by amplifying individual threonine biosynthesis genes and investigating the effect on the L-threonine production.

Object of the Invention

The inventors had the object of providing new measures for improved fermentative preparation of L-threonine.

Description of the Invention

The invention provides a polypeptide from Enterobacteriaceae with malate:quinone oxidoreductase (Mqo) activity (E.C. 1.1.99.16) chosen from the group consisting of

a) polypeptide with the amino acid sequence shown in SEQ ID NO. 2, or

b) polypeptide which is at least 70%, preferably at least 80%, particularly preferably at least 90 to 95% identical to the amino acid sequence shown in SEQ ID NO. 2, or

c) polypeptide according to SEQ ID NO. 2, including deletion, insertion or exchange of one or more amino acids, or

d) polypeptide according to SEQ ID NO. 2, including N- or C-terminal lengthening by one or more amino acids,

the total length of the polypeptide according to b), c) or d) being at least 514 and at most 544, preferably at least 519 and at most 539, in a preferred form at least 524 and at most 534, particularly preferably at least 527 and at most 531 amino acid radicals.

The invention furthermore provides a polynucleotide from Enterobacteriaceae which codes for a polypeptide with malate:quinone oxidoreductase (Mqo) activity (E.C. 1.1.99.16), chosen from the group consisting of

a) DNA which contains the nucleotide sequence corresponding to nucleobases 7 to 1593 of SEQ ID NO. 1, or

b) DNA according to a) corresponding to the degeneration of the genetic code, or

c) DNA according to a) containing sense mutations of neutral function, or

d) DNA which is at least 70%, preferably at least 80%, particularly preferably at least 90 to 95% identical to that mentioned in a) or b), or

e) polynucleotide which hybridizes with the DNA according to a), b), c) or d).

The invention also provides

- a DNA which is capable of replication and codes for the polypeptide shown in SEQ ID NO. 2,
- a vector containing the mqo gene corresponding to nucleobases 7 to 1593 of SEQ ID NO. 1, in particular plasmid pMW218mqo shown in figure 1.

"Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

"Polypeptides" is understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

The polypeptides according to the invention include the polypeptides according to SEQ ID NO. 2, which have malate:quinone oxidoreductase activity, and also those which are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical to the polypeptide according to SEQ ID NO. 2 and have the activity mentioned.

Finally, the invention provides a process for the fermentative preparation of L-threonine using Enterobacteriaceae which in particular already produce L-threonine and in which the nucleotide sequence(s) which code(s) for the mqo gene are enhanced, in particular over-expressed.

In particular, the process is a process for the preparation of L-threonine, which comprises carrying out the following steps:

a) fermentation of microorganisms of the family Enterobacteriaceae in which at least the mqo gene is enhanced (over-expressed), optionally in combination with further genes,

b) concentration of the L-threonine in the medium or in the cells of the microorganisms of the family Enterobacteriaceae, and

c) isolation of the L-threonine.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or a gene which codes for a corresponding enzyme or protein with a high activity, and optionally combining these measures.

The microorganisms which the present invention provides can prepare L-threonine from glucose, sucrose, lactose, fructose, maltose, molasses, starch, or from glycerol and ethanol. They are representatives of Enterobacteriaceae, in particular of the genera Escherichia and Serratia. Of the genus Escherichia the species E. coli and of the genus Serratia the species Serratia marcescens are to be mentioned in particular.

Suitable L-threonine-producing strains of the genus Escherichia, in particular of the species E. coli, are, for example

Escherichia coli TF427
Escherichia coli H4578
Escherichia coli KY10935

Escherichia coli EL1003

Escherichia coli VNIIgenetika MG-442
Escherichia coli VNIIgenetika VL334/pYN7
Escherichia coli VNIIgenetika M1
Escherichia coli VNIIgenetika 472T23
Escherichia coli VNIIgenetika TDH-6
Escherichia coli BKIIM B-3996
Escherichia coli BKIIM B-5318
Escherichia coli B-3996-C43
Escherichia coli B-3996-C80
Escherichia coli B-3996/pTWV-pps
Escherichia coli B-3996(pMW::THY)
Escherichia coli B-3996/pBP5

Escherichia coli kat 13

Escherichia coli KCCM-10132

Suitable L-threonine-producing strains of the genus Serratia, in particular of the species Serratia marcescens, are, for example

Serratia marcescens HNr21
Serratia marcescens TLr156
Serratia marcescens T2000.

The nucleotide sequence of the chromosome of E. coli is known and is available in databanks accessible to the public, such as, for example, the databank of the European Molecular Biology Laboratories (EMBL, Heidelberg, Germany).

Examples of such sequences deposited are the entries accessible under number AE000310 or D90850.

In the work on the present invention it was possible to identify the mqo gene, which codes for malate:quinone oxidoreductase, of E. coli (SEQ ID NO. 1) and the amino acid sequence of the Mqo·enzyme protein formed (SEQ ID NO. 2).

It has furthermore been possible to isolate two further new malate:quinone oxidreductase proteins, designated protein B and C, which have the N-terminal amino acid sequence shown in SEQ ID No. 11 and 12. These are also provided by the invention.

It has been found that Enterobacteriaceae produce L-threonine in an improved manner after over-expression of the mqo gene, which codes for malate:quinone oxidoreductase (E.C. 1.1.99.16).

According to the invention, it is also possible to use a DNA section which contains the DNA sequence of the gene of the malate:quinone oxidoreductase given in the databank of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) with accession number P33940.

Alleles of the mqo gene which result from the degeneracy of the genetic code or due to "sense mutations" of neutral function can furthermore be used. It is also known that the amino acid methionine or formylmethionine coded by the start codon ATG can be removed in various proteins by enzymes of the host.

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is

additionally possible to increase the expression in the course of fermentative L-threonine production. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructions can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Chang and Cohen (Journal of Bacteriology 134:1141-1156 (1978)), in Hartley and Gregori (Gene 13:347-353 (1981)), in Amann and Brosius (Gene 40:183-190 (1985)), in de Broer et al. (Proceedings of the National (sic) of Sciences of the United States of America 80:21-25 (1983)), in LaVallie et al. (BIO/TECHNOLOGY 11, 187-193 (1993)), in WO 98/04715, in Llosa et al. (Plasmid 26:222-224 (1991)), in Quandt and Klipp (Gene 80:161-169 (1989)), in Hamilton (Journal of Bacteriology 171:4617-4622 (1989), in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998) and in known textbooks of genetics and molecular biology.

Plasmid vectors which are capable of replication in Enterobacteriaceae, such as e.g. cloning vectors derived from pACYC184 (Bartolomé et al.; Gene 102, 75-78 (1991)), pTrc99A, which is described by Amann et al. (Gene 69:301-315 (1988)), or pSC101 derivatives (Vocke and Bastia, Proceedings of the National Academy of Science, USA 80 (21):6557-6561 (1983)) can be used. A strain transformed with a plasmid vector where the plasmid vector carries the nucleotide sequence which codes for the mqo gene can be employed in a process according to the invention.

In addition, it may be advantageous for the production of L-threonine with strains of the family Enterobacteriaceae to enhance, in particular to over-express, one or more enzymes of the known threonine biosynthesis pathway or enzymes of anaplerotic metabolism or enzymes for the production of reduced nicotinamide adenine dinucleotide phosphate, in addition to the mqo gene.

Thus, for example, one or more genes chosen from the group consisting of

- the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase (US-A-4,278,765),
- the pyc gene which codes for pyruvate carboxylase (DE-A-19 831 609),
- the pps gene which codes for phosphoenol pyruvate synthase (Molecular and General Genetics 231:332 (1992)),
- the ppc gene which codes for phosphoenol pyruvate carboxylase (Gene 31:279-283 (1984)),
- the genes pntA and pntB which code for transhydrogenase (European Journal of Biochemistry 158:647-653 (1986)),
- the rhtB gene which imparts homoserine resistance (EP-A-0994190)
- the rhtC gene which imparts threonine resistance (EP-A-1013765), and
- the gdhA gene which codes for glutamate dehydrogenase (Gene 27:193-199 (1984))

can be enhanced, in particular over-expressed, at the same time.

It may furthermore be advantageous for the production of L-threonine, in addition to the enhancement of the mqo gene, for one or more of the genes chosen from the group consisting of:

- the tdh gene which codes for threonine dehydrogenase (Ravnikar and Somerville, Journal of Bacteriology 169, 4716-4721 (1987)),
- the mdh gene which codes for malate dehydrogenase (E.C. 1.1.1.37)

to be attenuated, in particular to be eliminated or for the expression thereof to be reduced at the same time.

Finally, in addition to enhancement of the mqo gene it may be advantageous for the production of L-threonine to eliminate undesirable side reactions, (Nakayama: "Breeding of Amino Acid Producing Microorganisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982). Bacteria in which the metabolic pathways which reduce the formation of L-threonine are at least partly eliminated can be employed in a process according to the invention.

The microorganisms produced according to the invention can be cultured in the batch process (batch culture) or in the fed batch process (feed process). A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained

in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and optionally cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulphate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed

in a suitable manner to control the pH. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 25°C to 45°C, and preferably 30°C to 40°C. Culturing is continued until a maximum of L-threonine has formed. This target is usually reached within 10 hours to 160 hours.

The analysis of L-threonine can be carried out by anion exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190), or it can take place by reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

A pure culture of the L-threonine-producing strain B-3996kurΔtdh/pVIC40, pMW218mqo was deposited on 24th January 2001 at the Deutsche Sammlung für Mikroorgansimen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) as DSM 14004.

The process according to the invention is used for the fermentative preparation of amino acids, in particular L-threonine and L-isoleucine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

The isolation of plasmid DNA from E. coli and all techniques of restriction, Klenow and alkaline phosphatase treatment are carried out by the method of Sambrook et al. (Molecular cloning - A laboratory manual (1989) Cold Spring Harbour Laboratory Press). Unless described otherwise, the

transformation of E. coli is carried out by the method of Chung et al. (Proceedings of the National Academy of Sciences of the United States of America USA (1989) 86:2172-2175).

The incubation temperature during preparation of strains and transformants is 37°C. Temperatures of 30°C and 44°C are used in the gene replacement process according to Hamilton et.al.

Example 1

Construction of the expression plasmid pMW218mqo

The mqo gene from E. coli K12 is amplified using the polymerase chain reaction (PCR) and synthetic oligonucleotides. Starting from the nucleotide sequence of the yojH gene in E. coli K12 MG1655 (EMBL AE000310), PCR primers (see SEQ ID No. 3 and 4) are synthesized (MWG Biotech, Ebersberg, Germany):

YojH1: 5' - GCGGAATTCGATGGCGGCAAAAGCG - 3'

YojH2: 5' - GTTACGCCGCATCCAACATC - 3'

The chromosomal E. coli K12 MG1655 DNA employed for the PCR is isolated according to the manufacturers instructions with "QIAGEN Genomic-tips 100/G" (QIAGEN, Hilden, Germany). A DNA fragment approx. 1700 base pairs (bp) in size can be amplified with the specific primers under standard PCR conditions (Innis et al. (1990) PCR protocols. A guide to methods and applications, Academic Press) with Pfu-DNA polymerase (Promega Corporation, Madison, USA). The PCR product is cleaved with the enzyme EcoRI and ligated with the plasmid pMW218 (Nippon Gene, Toyama, Japan), which is cleaved with the enzymes EcoRI and SmaI. The E. coli strain DH5α is transformed with the ligation batch and plasmid-carrying cells are selected on LB agar (Lennox, Virology 1:190 (1955)), to which 20 µg/ml kanamycin is

added. Successful cloning of the mqo gene can be demonstrated after plasmid DNA isolation and control cleavage with EcoRI, AccI and ClaI. The plasmid is designated pMW218mqo (figure 1).

Example 2

Preparation of L-threonine with the strain MG442/pMW218mqo

2.1 Preparation of the strain MG442/pMW218mqo

The L-threonine-producing E. coli strain MG442 is described in US-A- 4,278,765 and deposited as CMIM B-1628 at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

The strain MG442 is transformed with the plasmid pMW218mqo and plasmid-carrying cells are selected on LB agar supplemented with 20 µg/ml kanamycin. The strain is designated MG442/pMW218mqo.

2.2 Preparation of L-threonine

Selected individual colonies of MG442/pMW218mqo are multiplied further on minimal medium with the following composition: 3.5 g/l $Na_2HPO_4*2H_2O$, 1.5 g/l KH_2PO_4, 1 g/l NH_4Cl, 0.1 g/l $MgSO_4*7H_2O$, 25 mg/l isoleucine, 2 g/l glucose, 20 g/l agar, 20 mg/l kanamycin. The formation of L-threonine is checked in batch cultures of 10 ml contained in 100 ml conical flasks. For this, 10 ml of preculture medium of the following composition: 2 g/l yeast extract, 10 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4*7H_2O$, 15 g/l $CaCO_3$, 20 g/l glucose, 20 mg/l kanamycin are inoculated and the batch is incubated for 16 hours at 37°C and 180 rpm on an ESR incubator from Kühner AG (Birsfelden, Switzerland). In each case 250 µl of this preculture are transinoculated into 10 ml of production medium (25 g/l $(NH_4)_2SO_4$, 2 g/l KH_2PO_4, 1 g/l $MgSO_4*7H_2O$, 0.03 g/l $FeSO_4*7H_2O$, 0.018 g/l $MnSO_4*1H_2O$, 25 mg/l isoleucine, 30 g/l $CaCO_3$, 20 g/l

glucose) and the batch is incubated for 48 hours at 37°C. After the incubation the optical density (OD) of the culture suspension is determined with an LP2W photometer from the company Dr. Lange (Berlin, Germany) at a measurement wavelength of 660 nm.

The concentration of L-threonine formed is then determined in the sterile-filtered culture supernatant with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column reaction with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

Strain	OD (660 nm)	L-Threonine g/l
MG442	4.7	0.46
MG442/pMW218mqo	4.5	0.89

Example 3

Preparation of L-threonine with the strain B-3996kurΔtdh/pVIC40, pMW218mqo

The L-threonine-producing E. coli strain B-3996 is described in US-A- 5,175,107 and deposited at the Russian National Collection for Industrial Microorganisms (VKPM, Moscow, Russia).

3.1 Preparation of the strain B-3996kurΔtdh/pVIC40, pMW218mqo

After culture in antibiotic-free complete medium for approximately ten generations, a derivative of strain B-3996 which no longer contains the plasmid pVIC40 is

isolated. The strain formed is streptomycin-sensitive and is designated B-3996kur.

The method described by Hamilton et al. (Journal of Bacteriology (1989) 171: 4617-4622), which is based on the use of the plasmid pMAK705 with a temperature-sensitive replicon, is used for incorporation of a deletion into the tdh gene. The plasmid pDR121 (Ravnikar and Somerville, Journal of Bacteriology (1987) 169:4716-4721) contains a DNA fragment from E. coli 3.7 kilo-base pairs (kbp) in size, on which the tdh gene is coded. To generate a deletion of the tdh gene region, pDR121 is cleaved with the restriction enzymes ClaI and EcoRV and the DNA fragment 5 kbp in size isolated is ligated, after treatment with Klenow enzyme. The ligation batch is transformed in the E. coli strain DH5α and plasmid-carrying cells are selected on LB agar, to which 50 μg/ml ampicillin are added.

Successful deletion of the tdh gene can be demonstrated after plasmid DNA isolation and control cleavage with EcoRI. The EcoRI fragment 1.7 kbp in size is isolated, and ligated with the plasmid pMAK705, which is partly digested with EcoRI. The ligation batch is transformed in DH5α and plasmid-carrying cells are selected on LB agar, to which 20 μg/ml chloramphenicol are added. Successful cloning is demonstrated after isolation of the plasmid DNA and cleavage with EcoRI. The pMAK705 derivative formed is designated pDM32.

For the gene replacement, B-3996kur is transformed with the plasmid pDM32. The replacement of the chromosomal tdh gene with the plasmid-coded deletion construct is carried out by the selection process described by Hamilton et al. and is verified by standard PCR methods (Innis et al. (1990), PCR Protocols. A Guide to Methods and Applications, Academic Press) with the following oligonucleotide primers (see SEQ ID No. 5 and 6).

Tdh1: 5'-TCGCGACCTATAAGTTTGGG-3'

Tdh2: 5'-AATACCAGCCCTTGTTCGTG-3'.

The strain formed is tested for kanamycin sensitivity and is designated B-3996kurΔtdh.

B-3996kurΔtdh is transformed with the plasmid pVIC40 isolated from B-3996 and plasmid-carrying cells are selected on LB agar with 20 µg/ml streptomycin. A selected individual colony is designated B-3996kurΔtdh/pVIC40 and transformed with the plasmid pMW218mqo. Selection is carried out on LB-agar to which 20 µg/ml streptomycin and 50 µg/ml kanamycin are added. The strain formed in this way is designated B-3996kurΔtdh/pVIC40, pMW218mqo.

3.2 Preparation of L-threonine

The preparation of L-threonine by the strains B-3996kurΔtdh/pVIC40 and B-3996kurΔtdh/pVIC40, pMW218mqo is tested as described in example 2, the minimal medium and the production medium not being supplemented with L-isoleucine. The minimal medium, the preculture medium and the production medium are supplemented with 20 µg/ml streptomycin for B-3996kurΔtdh/pVIC40 and with 20 µg/ml streptomycin and 50 µg/ml kanamycin for B-3996kurΔtdh/pVIC40, pMW218mqo.

The result of the experiment is summarized in Table 2.

Table 2

Strain	OD (660 nm)	L-Threonine
B-3996kurΔtdh/pVIC40	4.7	6.26
B-3996kurΔtdh/pVIC40, pMW218mqo	3.7	7.72

Example 4

Preparation of a vector containing a histidine-tagged malate:quinone oxidoreductase gene of E. coli

A 1744 bp DNA fragment, which codes for the malate:quinone oxidoreductase protein extended by a six-fold histidine radical on the C-terminal end, was prepared by means of the polymerase chain reaction (PCR) and then cloned.

The primer YOJH1a (SEQ ID No 7) was drafted with the aid of the known nucleotide sequence with Accession Number AE000310 (EMBL, European Molecular Biology Laboratories, Heidelberg, Germany). This primer has the sequence:

5`-GGA TCC GTT GAT GCC GCG CAA ATC-3`.

The primer YCHIS (SEQ ID No 8), which has the following sequence, was employed as the second primer:

5`-CGC GAA TTC TTA GTG GTG GTG GTG GTG GTG CAA CGC AAT ATC CGC CAC-3`.

The primers shown were synthesized by MWG Biotech (Ebersberg, Germany). The PCR reaction was carried out by the standard PCR method of Innis et al., (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press, New York, USA).

Whole DNA isolated from a colony of the E. coli strain MC4100 (Casadaban et al. Journal of Molecular Biology 104, 541-555, 1976) served as the template.

The PCR was carried out in a Thermocycler from Techne (Cambridge, UK). The samples were first denatured for 5 minutes at 94°C and the Taq polymerase from Promega (Madison, WI, USA) was then added to the sample batch. A cycle comprising denaturing (60 seconds, 94°C), annealing (60 seconds, 60°C) and synthesis (120 seconds, 72°C) was then passed through 10 times, the annealing temperature

being increased by 0.4°C in each cycle. The subsequent 25 cycles comprised denaturing (60 seconds, 94°C), annealing (60 seconds, 64°C) and synthesis (120 seconds, 72°C). Finally, a concluding synthesis of 10 minutes at 72°C was carried out.

The DNA fragment 1744 bp in length containing the mqo gene amplified in this manner was purified with the aid of the QIAQuick PCR Purification Kit from Qiagen (Hilden, Germany) and then digested with the restriction enzymes BamHI and EcoRI. These restriction cleavage sites were generated during the PCR with the aid of the primers YOJHI and YCHIS. After gel electrophoresis, the digested DNA fragment was cut out of the agarose gel and purified with QIAEX II Gel Extraction Kit (155) (Hilden, Germany), mixed into the vector pUC19 treated with the restriction enzymes BamHI and EcoRI (Yanisch-Perron et al., Gene 33, 103-119, 1985) and then treated with T4 DNA ligase.

An E. coli strain designated MC4100Δmqo, which contains a deletion in the mqo gene and was prepared according to the prior art, was used as the cloning host for the transformation. For this, the mqo gene was first amplified with the aid of the primers Y_01 (SEQ ID No 9) and Y_04 (SEQ ID No 10) using whole DNA isolated from strain MC4100, with the aid of the PCR method The PCR conditions comprised 30 cycles of denaturing (30 seconds, 94°C), annealing (30 seconds, 60°C) and synthesis (2 minutes, 72°C).

The primer Y_01 has the following sequence:
5`-GCTGGATGAATGGGCGGCGG-3`

The primer Y_04 has the following sequence:
5`-CGC<u>GGATCC</u>CCGGTTTCAACGATGATG-3`

The amplified DNA fragment contains a cleavage site for the restriction enzyme BamHI directly after the primer Y_01.

The BamHI restriction cleavage site contained in the oligonucleotide primer Y_04 is identified by underlining. The amplified DNA fragment was digested with BamHI and then incorporated into the BamHI cleavage site of the plasmid pKO3 described by Link et al. (Journal of Bacteriology 179, 6228-6237 (1997)). The resulting plasmid was designated pKO3mqo and treated with the restriction enzyme MluI in order to remove an internal DNA segment of the mqo gene 416 bp long (deletion). The plasmid pKO3Δmqo obtained in this manner was used for incorporation of the deletion Δmqo in the strain MC4100. The method described by Link et al (Journal of Bacteriology 179, 6228-6237 (1997)) was employed for this.

The strain MC4100Δmqo was transformed with the ligation mixture described above. Transformants were selected on LB medium, which had been supplemented with 100µg/ml carbenicillin. A plasmid was isolated from a transformant and designated pUCH2. Plasmid pUCH2 contains a DNA fragment 1744 bp long, which codes for the malate:quinone oxidoreductase protein extended by a six-fold histidine radical on the C-terminal end.

Example 5

Isolation and purification of the over-expressed histidine-tagged malate:quinone oxidoreductase

Five times, 200 ml LB medium were treated with 100 µg/ml carbenicillin and 100 µM isopropyl β-D-thiogalactoside (IPTG), inoculated with in each case a colony of the strain MC4100Δmqo/pUCH2 and in each case cultured in 1 l conical flasks for 16 hours at 37°C and 200 revolutions per minute. The cells were washed twice in buffer A (50 mM hepes, 10 mM potassium acetate, 10 mM $CaCl_2$, 5 mM $MgCl_2$, adjusted to pH 7.5 with NaOH) at 4°C and resuspended in 40 ml of the same buffer. The cells were then broken down twice in a precooled French Pressure Cell from Spectronic Unicam

(Rochester, NY, USA) under 69 MPa (mega-Pascal). The cell debris was then sedimented twice in a centrifuge at 4°C for 10 minutes at 10000 x g. The supernatant was then centrifuged for 30 minutes at 75000 x g and 4°C. The membrane pellet was resuspended with the same volume of buffer B (50 mM Na phosphate, 200 mM NaCl, pH 7.5) and centrifuged again for 30 minutes at 75000 x g and 4°C. The pellet was then resuspended with 1 ml buffer B. The histidine-tagged malate:quinone oxidoreductase protein was purified in two steps.

Step 1: Solubilization:

2 % Triton X-100 and 10 % glycerol were added to the resuspended membranes and the batch was incubated for 10 minutes on ice. The batch was then centrifuged for 30 minutes at 200000 x g at 4°C.

Step 2: Affinity chromatography:

The equilibration of the "Talon-Metal-Affinity Resin" column material (500 µl column volume, CLONTECH Laboratories, Palo Alto, USA) was carried out twice with 1 ml buffer B and once with 1 ml buffer C (50 mM Na phosphate, 200 mM NaCl, 0.05 % Triton X-100, 10 µM flavin adenine dinucleotide (FAD), 0.2 mg/ml phospholipid, pH 7.0). The phospholipid used was L-α phosphatidylethanolamine, type IX from E. coli (Sigma-Aldrich, Deisenhofen, Germany), which was mixed as a 30 mg/ml stock solution in deionized water and treated briefly with an ultrasound apparatus (BRANSON Sonifier Cell Disrupter B15) for a few seconds until the suspension was transparent. The supernatant (1 ml) from step 1 was applied to the equilibrated column and incubated for 20 minutes at room temperature. Thereafter, the column was flushed five times with buffer D (50 mM Na phosphate, 200 mM NaCl, 0.05 % Triton X-100, 10 % glycerol, 10 µM FAD, 0.2 mg/ml phospholipid, 10 mM imidazole, pH 7.0) and then

eluted twice with 500 µl buffer E (50 mM Na phosphate, 200 mM NaCl, 0.05 % Triton X-100, 10 % glycerol, 10 µM FAD, 0.2 mg/ml phospholipid, 100 mM imidazole, pH 7.0). The two fractions were combined and a buffer exchange was carried out by means of an ULTRAFREE-0.5 Centrifugal Filter Device (Millipore Corporation, Bedford, MA, USA), in order to remove the imidazole and to reduce the volume to 500 µl. A second affinity chromatography was then carried out with the "Talon-Metal-Affinity Resin" column material (250 µl column volume), as described above. The purified protein was stored at -20°C.

The purified malate:quinone oxidoreductase protein was investigated by means of SDS polyacrylamide gel electrophoresis and subsequent staining with Coomassie blue. In this analysis, two protein bands (protein B and protein C) with the mobility corresponding to a molecular weight of about 60 ± 2 KD (kilo-Dalton) were detected. The two proteins were blotted on to a polyvinylidene difluoride (PVDF) membrane (Boehringer Mannheim, Mannheim, Germany) and stained with Coomassie blue. The two protein bands were then cut out of the blot membrane.

<u>Example 6</u>

Determination of the N-position amino acid sequence

The N-position amino acid sequences of the malate:quinone oxidoreductase protein B and protein C were determined by Edman degradation (Edman, Molecular Biology Biochemistry Biophysics 8:211-55(1970)) by means of the automatic sequencer Procise Sequencer from PE Biosystems (Foster City, CA, USA). For protein B the amino acid sequence L N A V S M (see also SEQ ID No. 11) and for protein C the amino acid sequence A V S M A A K (see also SEQ ID No. 12) was determined.

Brief Description of the Figures:

Figure 1: Map of the plasmid pMW218mqo containing the mqo gene.

The length data are to be understood as approx. data. The abbreviations and designations used have the following meaning:

- Plac: Promoter sequence of the lactose operon
  Kan: Kanamycin resistance gene

The abbreviations for the restriction enzymes have the following meaning

- AccI: Restriction endonuclease from Acinetobacter calcoaceticus
- ClaI: Restriction endonuclease from Caryphanon latum
- EcoRI: Restriction endonuclease from E. coli
- KpnI: Restriction endonuclease from Klebsiella pneumoniae
- SalI: Restriction endonuclease from Streptomyces albus

What is claimed is:

1. A polypeptide from Enterobacteriaceae with malate:quinone oxidoreductase (Mqo) activity (E.C. 1.1.99.16) chosen from the group consisting of

   a) polypeptide with the amino acid sequence shown in SEQ ID NO. 2, or

   b) polypeptide which is at least 70%, preferably at least 80%, particularly preferably at least 90 to 95% identical to the amino acid sequence shown in SEQ ID NO. 2, or

   c) polypeptide according to SEQ ID NO. 2, including deletion, insertion or exchange of one or more amino acids, or

   d) polypeptide according to SEQ ID NO. 2, including N- or C-terminal lengthening by one or more amino acids,

   the total length of the polypeptide according to b), c) or d) being at least 514 and at most 544, preferably at least 519 and at most 539, in a preferred form at least 524 and at most 534, particularly preferably at least 527 and at most 531 amino acid radicals.

2. A polynucleotide from Enterobacteriaceae which codes for a polypeptide with malate:quinone oxidoreductase (Mqo) activity (E.C. 1.1.99.16), chosen from the group consisting of

   a) DNA which contains the nucleotide sequence corresponding to nucleobases 7 to 1593 of SEQ ID NO. 1, or

   b) DNA according to a) corresponding to the degeneration of the genetic code, or

c) DNA according to a) containing sense mutations of neutral function, or

d) DNA which is at least 70%, preferably at least 80%, particularly preferably at least 90 to 95% identical to that mentioned in a) or b), or

e) polynucleotide which hybridizes with the DNA according to a), b), c) or d).

3. A polynucleotide as claimed in claim 2, which is DNA which is capable of replication and codes for the polypeptide shown in SEQ ID NO. 2.

4. The plasmid pMW218mqo which contains the mqo gene of Escherichia coli.

5. A process for the fermentative preparation of L-threonine, which comprises employing Enterobacteriaceae bacteria, in particular those which already produce L-threonine and in which the nucleotide sequence(s) which code(s) for the mqo gene are enhanced, in particular over-expressed.

6. A process as claimed in claim 5, wherein further genes are enhanced in addition to the mqo gene.

7. A process as claimed in claim 5 or 6, wherein the microorganisms of the family Enterobacteriaceae are from the genus Escherichia and Serratia.

8. A process as claimed in claim 7, wherein the microorganisms are from the genus Escherichia, in particular of the species Escherichia coli.

9. A process as claimed in claim 5, wherein the thrABC operon which codes for aspartate kinase, homoserine dehydrogenase, homoserine kinase and threonine synthase is enhanced at the same time.

10. A process as claimed in claim 5, wherein the pyc gene which codes for pyruvate carboxylase is enhanced at the same time.

11. A process as claimed in claim 5, wherein the pps gene which codes for phosphoenol pyruvate synthase is enhanced at the same time.

12. A process as claimed in claim 5, wherein the ppc gene which codes for phosphoenol pyruvate carboxylase is enhanced at the same time.

13. A process as claimed in claim 5, wherein the genes pntA and pntB which code for transhydrogenase are enhanced at the same time.

14. A process as claimed in claim 5, wherein the gene rhtB which imparts homoserine resistance is enhanced at the same time.

15. A process as claimed in claim 5, wherein bacteria in which the metabolic pathways which reduce the formation of L-threonine are at least partly eliminated are employed.

16. A process as claimed in claim 5, wherein a strain transformed with a plasmid vector is employed and the plasmid vector carries the nucleotide sequence which codes for the mqo gene can be employed.

17. A process as claimed in claim 5, wherein bacteria transformed with the plasmid pMW218mqo are employed.

18. A process as claimed in claim 5, wherein the expression of the mqo gene is optionally induced with isopropyl β-D-thiogalactoside.

19. A process as claimed in claim 5, wherein at the same time the gdhA gene which codes for glutamate dehydrogenase is enhanced.

20. A process as claimed in claim 5, wherein at the same time the rhtC gene which imparts threonine resistance is enhanced.

21. A process as claimed in claim 5, wherein the nucleotide sequence codes for a malate:quinone oxidoreductase protein with the N-terminal amino acid sequence Met Ala Ala Lys Ala Lys corresponding to SEQ ID No. 2.

22. A process as claimed in claim 5, wherein the nucleotide sequence codes for a malate:quinone oxidoreductase protein with the N-terminal amino acid sequence Leu Asn Ala Val Ser Met according to SEQ ID no. 11.

23. A process as claimed in claim 5, wherein the nucleotide sequence codes for a malate:quinone oxidoreductase protein with the N-terminal amino acid sequence Ala Val Ser Met Ala Ala Lys according to SEQ ID No. 12.

24. A process for the preparation of L-threonine, which comprises carrying out the following steps:

    a) fermentation of microorganisms of the family Enterobacteriaceae in which at least the mqo gene is enhanced (over-expressed), optionally in combination with further genes,

    b) concentration of the L-threonine in the medium or in the cells of the microorganisms of the family Enterobacteriaceae, and

    d[sic]) isolation of the L-threonine.

25. A malate-quinone oxidoreductase protein from Enterobacteriaceae with the N-terminal amino acid sequence according to SEQ ID No. 11.

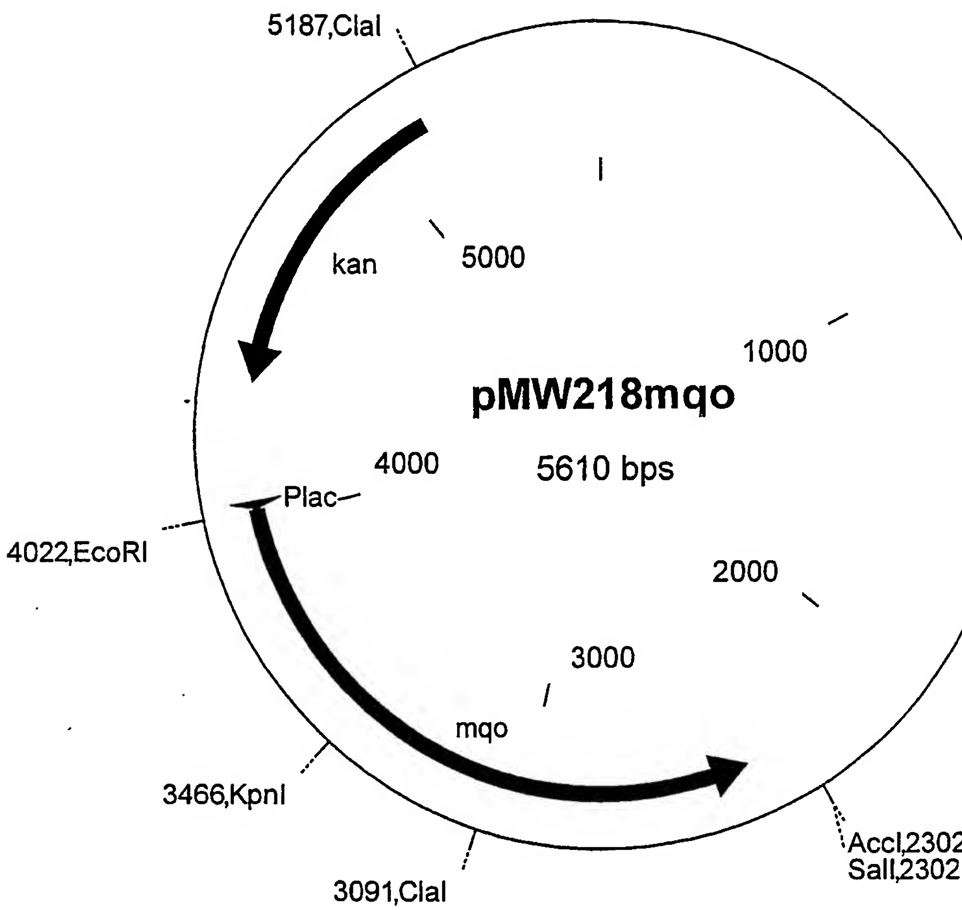
26. A malate:quinone oxidoreductase protein from Enterobacteriaceae with the N-terminal amino acid sequence according to SEQ ID No. 12.

27. An L-threonine-producing strain of the genus Escherichia with the genetic and phenotypic features of the strain B-3996kurΔtdh/pVIC40, pMW218mqo.

28. The L-threonine-producing Escherichia coli strain B-3996kurΔtdh/pVIC40, pMW218mqo deposited as.(sic) DSM 14004.

1/1

Figure 1: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Lys Val Ala Ala Thr Arg Thr Glu Ile Gly Thr Asp Val Asn Tyr Gly
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/05548

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/02 C12P13/08 C12N15/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, WPI Data, PAJ, EPO-Internal, EMBL, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KATHER BIRGIT ET AL: "Another unusual type of citric acid cycle enzyme in Helicobacter pylori: The malate:quinone oxidoreductase." JOURNAL OF BACTERIOLOGY, vol. 182, no. 11, June 2000 (2000-06), pages 3204-3209, XP002179952 ISSN: 0021-9193	1-4,25, 26
Y	tables 1,2	5-24,27, 28
X	DATABASE EMBL 'Online! BLATTNER F.R. ET AL.: "The complete genome sequence of Escherichia coli K-12." retrieved from EBI Database accession no. P33940 XP002179954 the whole document	1-4,25, 26

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[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search
19 October 2001

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